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THE USE OF TISSUE IN BROTH IN THE PRODUCTION OF DIPHTHERIA TOXIN

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The production of diphtheria toxin regularly and of sufficiently high potency has long been a matter of primary importance. Although diphtheria toxin was among the first of the bacterial products to be discovered, but little is now known as to its composition or the basic substances from which it is produced. The well known uncertainty of toxin production and the seasonal variation in potency (MacConkey [1]) emphasize the importance of finding a method of producing toxin regularly and of satisfactory strength. A study of the literature on the nature of diphtheria toxin fails to supply much definite information.

An excellent résumé of the literature on the nature of this substance is contained in the paper by Robinson and Rettger.[2] It is of interest to mention a few of the conclusions of investigators having some bearing on the work which we are to report. It has been generally agreed that peptone was the important factor in enabling the diphtheria bacilli to produce toxin, Hida[3] believing that the secondary proteoses were the important constituents. Hitchens[4] and others found that simple peptone solution does not support the production of toxin. Dzierzgowski and Rekowski[5] believed that the toxin was a combination of certain bases and albumoses, as no toxin is obtained until the medium turns alkaline. The latter point was recently confirmed by Bunker.[6] The work of Hadley[7] on the production of toxin from protein-free mediums is open to criticism, since a whole culture and not a bacterial free filtrate was used for toxicity tests. Spronk[8] replaced meat infusion by an extract of brewer's yeast and claimed that he obtained stronger toxin and greater regularity in its production. Smith[9] found that the oxygen supply was an important factor and observed that 0.2% dextrose was favorable to toxin production on account of its acceleration of growth. No acid was formed from glycogen.

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[1] Jour. Hygiene, 1912, 12, p. 507.
[2] Jour. Med. Res., 1917, 36, p. 357.
[3] Ztschr. f. Hyg., 1908, 61, p. 273.
[4] Jour. Med. Res., 1905, 13, p. 523.
[5] Quoted from Robinson and Rettger.
[6] Jour. Bacteriol., 1919, 4, p. 379.
[7] Jour. Infect. Dis., 1907, Supplement 3, p. 95.
[8] Ann. de L'Inst. Pasteur, 1898, 12, p. 701.
[9] Jour. Exper. Med., 1899, 4, p. 373.

In view of the lack of Witte peptone, the former standard for toxin production, it seemed desirable to utilize only American peptone, especially since such a product would be constantly available. The proteose peptone manufactured by the Digestive Ferments Company and the peptone of the Parke-Davis Company have given uniformly good results with the method to be described. None of the other peptones tested gave as favorable results.

The medium used by us is essentially the "hormone" broth of Huntoon[10] and is prepared as follows: Finely ground lean veal is infused in twice its weight of water and is stored in the icebox for from 18 to 24 hours. It is then filtered through a wire gauze and heated to 80 degrees for 5 minutes. The infusion is thoroughly cooled and any fat present is removed from the surface. Two per cent. peptone and 0.5% sodium chlorid are added. The medium is brought to the boiling point and held there for 5 minutes. It is then titrated, using phenolphthalein as an indicator, to from neutral to 0.3% acid and again boiled 5 minutes. If the reaction is found to be correct the broth is filtered through wire gauze and the sediment allowed to settle. It necessary, the reaction is again corrected before filtration. The medium is distributed in 125 c c amounts in 300 c c. Florence flasks and sterilized 15 minutes at 15 pounds pressure. The reaction of the medium and its relation to toxin production is discussed in detail in another section.

In view of the fact that Bull and Pritchett[11] found that B. welchii produced more toxin in the presence of fresh sterile tissue, it was considered advisable to determine the effect on diphtheria toxin production of adding tissue to broth prepared in the manner already described. Various tissues from the rabbit and guinea-pig have been used, but guinea-pig liver tissue gives by far the best results. Liver tissue from guinea-pigs which have been negative to toxin tests are satisfactory, as no difference in results has been noted between tissue derived from these animals and that from normal pigs. The liver tissue was introduced into the flasks of broth in the following manner: A large guinea-pig was killed by a blow on the head or at the base of the skull and plunged into a 0.5% lysol solution for a few minutes. The body cavity was then asceptically opened and a mass of liver slightly larger than a 25-cent piece was introduced into each flask of 125 c c of medium. The liver tissue was not exsanguinated. Freshly drawn guinea-pig blood neither enhances nor inhibits the production of toxin. It seemed desirable to use guinea-pig tissue in order to avoid foreign protein toxicity in the diphtheria toxin tests. It has been found that when guinea-pig liver tissue is removed with care, according to the technic just described, it is nearly uniformly sterile for less than 1 out of 10 pigs appears to be infected. The liver tissue was transferred directly from the animal into the flasks of sterilized mediums and inoculated immediately with the culture of diphtheria bacillus because tissue which has stood in mediums in the incubator to test its sterility loses its power of enhancing toxin production. The reason for this loss of enriching property is not clear. Contaminations of the mediums from infected tissue or other sources can be detected in smear preparations at the time of testing the toxin and also by the odor of the broth.

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[10] Jour. Infect. Dis., 1918, 23, p. 169.

[11] Jour. Exper. Med., 1917, 26, p. 119.

On account of the favorable results obtained by the addition of sterile liver tissue to broth, the effect of adding a water extract of beef liver tissue to broth in place of the tissue was tried but with negative results. Infusion broth prepared from beef liver instead of veal does not give toxin production comparable with the ordinary infusion broth to which guinea-pig liver has been added. Two explanations may be advanced for these results: (1) there may be some physical or mechanical reaction with the freshly excised liver tissue or (2) the essential substance is destroyed by sterilizing the liver infusion.

Inoculation of the tissue broth was made from fresh moist 24-hour cultures on Loeffler blood serum and luxuriant pellicle formation always resulted within 30 hours, provided the tissue was sterile. When the tissue was not sterile pellicle formation was not only greatly retarded, but the piece of tissue usually rose to the surface and floated, whereas sterile tissue invariably remained at the bottom of the flask. The Park-Williams strain No. 8 was used in all tests unless otherwise noted.

TABLE 1

THE EFFECT OF THE INITIAL REACTION ON TOXIN PRODUCTION

Initial Reaction	Incubation	Dilution of Toxin		Remarks
		0.01	0.005	
Neutral	4 days	Not tested	Pig died after 3 days	Heavy pellicle
Plus 0.1	4 days	Pig died after 2 days	Pig died after 2 days	Heavy pellicle in 36 hours
Plus 0.3	4 days	Not tested	Pig died after 2 days	Good pellicle
Plus 0.45	6 days	Pig died after 6 days	Pig died after 7 days	Pellicle formed slowly
Plus 0.5	5 days	Negative	Negative	Reaction in 0.01 pig
Plus 0.5	7 days	Pig died after 3 days	Negative	Pellicle formed in 48 hours
Plus 0.8	8 days	Negative	Not tested	Pellicle formed in 36 hours
Plus 1.0	8 days	Negative	Not tested	Poor pellicle formation
Neutral (control)	6 days	Negative	Negative	0.1 toxin produced

Since the purpose of this study was to find a method that would assure the regular production of toxin of usable strength rather than one which would supply an occasional high titer toxin interspersed with many nontoxin producing starts, a minimum lethal dose of 0.005 was set as a standard. Tests of considerably higher dilutions of tissue enriched broth were occasionally made and the results in most cases were positive. While not all the toxins produced were tested at a strength of 0.003 there is every evidence to show that most of them were at least of that titer while several had a titer greater than 0.0025. Broth prepared with Parke-Davis peptone and not enriched with liver tissue usually produced a low potency toxin with occasional toxins of a titer up to 0.005. Proteose peptone without tissue produced a 0.005 toxin or one of somewhat higher strength with greater regularity but in our hands 25% of the lots of medium prepared with this peptone failed to produce a 0.005 toxin.

As Bunker[6] has pointed out, the initial reaction of the broth determines to a considerable extent the potency of the toxin produced, as well as the character and speed of pellicle formation. Table 1 gives the initial reactions of different lots of broth varying from neutral to 1% acid to phenolphthalein.

Parke-Davis peptone was used in the preparation of each lot shown in this table. Tissue was used in the broth in each case unless otherwise indicated.

The reactions to phenolphthalein were checked by hydrogen-ion readings by the colorometric method and mediums showing reactions to phenolphthalein ranging from neutral to plus 0.3% fall within or near the limits indicated by Bunker.[6] It seems worthy of notice that equally satisfactory results are obtainable when the reaction of diphtheria toxin broth is adjusted by either the phenolphthalein or hydrogen-ion method. Table 1 indicates that in broth to which tissue has been added the most favorable initial reaction range to phenolphthalein is from neutral to plus 0.3%. As the reaction approaches plus 1.0%, toxin production becomes more and more irregular and finally ceases entirely. When toxin is formed in mediums with acid reactions of more than plus 0.3% to phenolphthalein the titer is lower than in mediums prepared at the same time and from the same veal infusion but with a reaction below plus 0.4%.

The initial reaction also appears to bear a fairly direct relation to the rate and luxuriance of pellicle formation for with mediums ranging from neutral to plus 0.3% a heavy, firm pellicle is formed within 24 hours and increases in thickness for about 3 days, after which it appears to remain constant. With a higher concentration of acid, pellicle formation is greatly retarded and the pellicles when finally formed are frequently incomplete or extremely thin and easily destroyed by slight agitation of the medium. The same conditions regarding the effect of the initial reaction of the medium on toxin production and pellicle formation seem to apply to proteose peptone as table 2 demonstrates.

TABLE 2

THE EFFECT OF THE INITIAL REACTION ON TOXIN PRODUCTION

Initial Reaction	Incubation	Dilution of Toxin		Remarks
		0.01	0.005	
Neutral	4 days	Pig died after 3 days	Pig died after 4 days	Heavy pellicle
Plus 0.2	4 days	Pig died after 2 days	Pig died after 5 days	Heavy pellicle
Plus 0.5	7 days	Negative	Negative	Slow pellicle formation
Plus 0.8	7 days	Not tested	Pig died after 8 days	Slow pellicle formation
Neutral (control)	4 days	Pig died after 4 days	Negative	Good pellicle formation
Neutral (control)	5 days	Not tested	Pig died after 2 days	Heavy pellicle

In testing the toxins shown in tables 1 and 2 due account was taken of the fact brought out by Bunker[6] that when the initial reaction is acid a longer incubation period must be allowed for the reversal to the alkaline reaction. It appears, however, that even when incubation is prolonged toxin is of low potency if the initial reaction varies to any great extent from the optimum.

Although the initial reaction of broth is of the greatest importance, it is equally necessary that the reaction become markedly alkaline if a high titer toxin is to be produced. Bunker[6] has already pointed out this fact and experiments with broth enriched with liver tissue confirm his observations. A tissue enriched flask seldom shows a reaction more alkaline than control flasks to which no tissue has been added. In the great majority of cases the reactions of the control flasks increase in alkalinity at a more rapid rate after the third day or sometimes the fourth day of incubation. This point is discussed later. Table 3 shows the initial reaction, the toxin production and the reaction at the time of testing of a lot of broth prepared with Parke-Davis peptone.

The interesting fact is brought out by this table that although the final hydrogen-ion reaction may be in a range where toxin is produced, nevertheless, if the initial reaction is too alkaline there is no toxin production. Here again the period of incubation was taken into account in order that sufficient time might be allowed for toxin production. When the reaction before testing has reached a value of P_H 8.0 one may expect to find a potent toxin in so far as this detail is concerned. It appears evident that toxin is only produced in broth which has a final reaction of at least P_H 8.0 and that stronger toxins usually have a more alkaline reaction up to P_H 8.3. A favorable reaction at the time of testing is not, however, an indication that toxin has been produced, for many lots of mediums without tissue enrichment had hydrogen-ion reactions within ranges where high titer toxins would be expected but with negative tests in guinea-pigs. It is evident that while the initial and final reaction of the medium has a direct bearing on toxin production other factors must also be favorable if the medium is to yield toxin.

TABLE 3

THE EFFECT OF INITIAL AND FINAL REACTION ON TOXIN PRODUCTION

Initial Reaction		Toxin Production		Reaction before Filtration, P_H
Phenolphthalein	P_H	0.01	0.005	
—0.1	7.9	Negative	Negative	8.0
Neutral	7.6	Died after 4 days	Died after 4 days	8.0
Neutral	7.6	Died after 4 days	Died after 4 days	8.0
Neutral	7.7	Not tested	Died after 3 days	8.2
Plus 0.4	7.3	Died after 3 days	Died after 3 days	8.3
Plus 0.4	7.2	Not tested	Died after 3 days	8.3
Neutral (control)	7.3	Died after 11 days	Negative	8.2
Neutral (control)	7.4	Died after 2 days	Died after 3 days	8.1

The addition of sterile liver tissue to broth appears to reduce the necessary period of incubation considerably. Most investigators have reported that incubation of not less than 6 days and usually longer is required. According to Bunker[6] this period is closely bound up with the progressive change in the reaction of the broth into an alkaline range. While this may be true, the constituents of the broth and particularly those substances that reduce the lag period and greatly enhance rapid growth, shorten the necessary incubation period for the production of a favorable alkaline reaction. When tissue is added to broth the optimum range of alkalinity appears to extend over a longer period of time than in unenriched mediums. Apparently the liver tissue has a buffer action independent of the peptone but this point has not been determined absolutely.

Diptheria bacilli grow with much greater rapidity in broth to which a piece of sterile liver tissue has been added than in unenriched mediums. Pellicle formation is far more luxuriant and the pellicle shows as a white film in from 18 to 24 hours, while a pellicle of equal strength and thickness is not formed on normal broth until from 30 to 36 hours have elapsed. The increase in strength and thickness of the pellicle after it is formed is more rapid when tissue has been added and at any time during incubation the enriched broth presents a more favorable pellicle for toxin production than control flasks of the same lot of medium. It is natural to expect that when growth and luxuriant pellicle formation are so rapid less time would be required for the production of a toxin of reasonably high potency. Table 4 gives the toxin production over a series of days of flasks filled with the same lot of broth and inoculated under as nearly the same conditions as possible. Parke-Davis peptone was used in the preparation of this broth and the reaction was neutral to phenolphthalein.

TABLE 4

Relation of the Period of Incubation to Toxin Production

Period of Incubation	Dilution of Toxin	
	0.01	0.005
4 days	Not tested	Died after 4 days
4 days (without tissue)	Negative	Negative
5 days	Not tested	Died after 5 days
5 days (without tissue)	Negative	Negative
6 days	Not tested	Died after 3 days
6 days (without tissue)	Negative	Negative
7 days	Not tested	Died after 2 days
8 days	Died after 2 days	Died after 3 days
8 days (without tissue)	Died after 2 days	Died after 3 days

TABLE 5

Relation of Initial Reaction, Rate and Potency of Toxin Production and Final Reaction

Period of Incubation	Dilution of Toxin		Final Reaction
	0.01	0.005	
3 days—tissue	Not tested	Died after 2 days	Ph 8.0
3 days—control	Not tested	Negative	Ph 7.9
4 days—tissue	Not tested	Died after 4 days	Ph 8.1
4 days—control	Not tested	Negative	Ph 8.1
5 days—tissue	Not tested	Died after 2 days	Ph 8.1
5 days—control	Died after 2 days	Died after 2 days	Ph 8.2
6 days—tissue	Not tested	Died after 3 days	Ph 8.2
6 days—control	Not tested	Negative	Ph 8.3
7 days—tissue	Not tested	Negative	Ph 8.3
7 days—control	Not tested	Negative	Ph 8.4

This series of tests was selected because one of the flasks of broth without tissue enrichment produced a 0.005 toxin and this fact allows a comparison with tissue enriched mediums in relation to speed of toxin production. Unfortunately the final reaction of this series of tests was not recorded, but similar series in which the Ph value was taken showed that broth enriched with sterile liver tissue attains a favorable alkaline reaction usually on the third day provided the initial reaction ranged from neutral to plus 0.3% to phenolphthalein as shown in table 5. The control flasks filtered on the fourth, fifth and sixth days did not contain a 0.01 toxin but on the eighth day a 0.005 toxin had been produced. It is evident from this table that broth without tissue enrichment requires longer incubation than flasks filled with the same lot of medium to which sterile pieces of liver tissue have been added. Table 5 brings out the relation between the initial reaction the rate and potency of toxin production and the final reaction. In this lot of broth proteose peptone was used and the initial reaction was neutral to phenolphthalein or Ph 7.7. A flask with tissue and a control were inoculated for each of the 5 days and tested as indicated.

This table presents clearly the advantage derived from adding tissue to broth which produces toxin. When the initial reaction is in the correct range for toxin production tissue enriched broth reaches a favorable alkalinity usually on the third or fourth day and the reaction continues advantageous over a considerable period of time. Furthermore, this table confirms the results recorded in table 4, for toxin production was more rapid in broth containing tissue than in the same lot of medium unenriched. When tissue is added to broth there appears to be less danger of collecting the toxin at an incorrect period of incubation since toxin is frequently found in tissue free broth on only one or two days during the whole course of the experiment.

The effect of an initial acid reaction on the rate and potency of toxin production is brought out in table 6. In this lot of broth proteose peptone was used and the initial reaction was plus 0.4 to phenolphthalein or P_H 7.2. A flask with tissue and a control were inoculated for each of the 5 days.

TABLE 6

The Effect of an Unfavorable Acid Reaction on Toxin Production

Period of Incubation	Dilution of Toxin		Final Reaction
	0.01	0.005	
4 days—tissue	Negative	Negative	P_H 8.0
4 days—control	Negative	Negative	P_H 7.9
5 days—tissue	Died after 2 days	Negative	P_H 8.2
5 days—control	Negative	Negative	P_H 8.1
6 days—tissue	Not tested	Died after 6 days	P_H 8.3
6 days—control	Negative	Negative	P_H 8.3
7 days—tissue	Died after 2 days	Died after 3 days	P_H 8.3
7 days—control	Negative	Negative	P_H 8.4
8 days—tissue	Not tested	Died after 7 days	P_H 8.4
8 days—control	Not tested	Died after 6 days	P_H 8.3

The same point is again brought out in this table as in table 3. The fact that the final reaction has reached a favorable degree of alkalinity is no guarantee that toxin has been produced. The effect of liver tissue on broth of poor toxin producing qualities is well shown in table 6. While the control flask of the eighth day of incubation contained a 0.005 toxin of poor quality, the flask with tissue enrichment on the seventh day showed a good quality, 0.005 toxin and one of the same strength but poorer quality on the sixth day. This table again confirms the results recorded in table 4, since toxin was produced with greater rapidity in broth containing sterile tissue.

Since the purpose of this study was to devise a method of producing diphtheria toxin regularly, little attention has been given to the exact potency of the product provided it was at least 0.005. Some of the control lots of broth, particularly that prepared with proteose peptone, produced toxins of a strength as high as 0.0033 and even 0.0025. In such cases the corresponding flask of broth with tissue added gave an equally high titer and within a shorter period of time. Occasionally a broth with tissue enrichment was tested at 0.0033, and in most cases was positive, although the control flask frequently did not contain a 0.01 toxin. Tests have been made on 18 lots of tissue enriched broth prepared with proteose or Parke-Davis peptone, the initial reaction ranging from neutral to plus 0.3% to phenolphthalein. In each of these lots a 0.005 toxin was obtained from flasks incubated from 3-7 days, the optimum period of incubation appearing to be about 5 days. Broth has been prepared by other methods than that previously described, but with unsatisfactory results. Flasks of mediums in which tissue was incubated 24 hours to test for sterility before inoculation with the culture of B. diphtheriae failed to produce toxin. Evidently, the liver tissue must be absolutely fresh to be of value in enhancing toxin production. Similar results were obtained by Bull and Pritchett[11] in their study of the toxin production of B. welchii.

Several strains of B. diphtheriae have been tested for toxin production by this method and encouraging results have been obtained. These cultures were isolated from active or convalescent cases, and had been cultivated on Loeffler blood serum slants for from 1-12 months. They had never been grown in broth up to one week before the tests were made when they were planted in tubes of "Hormone" broth to which small pieces of liver tissue had been added to encourage pellicle formation. After transferring a few times, pieces of the pellicles were transplanted onto the surface of broth in flasks after the tissue had been added. Eleven strains of B. diphtheriae were tested in this manner, of which but one failed to produce toxin. Eight of the remainder produced a 0.02 toxin, one had a strength of 0.006, while one killed in a dilution of 0.004. These tests still further support the belief that the addition of liver tissue to broth is of great value in the production of toxin.

The explanation of the effect of tissue on toxin production is extremely difficult owing to the lack of knowledge of the chemistry of tissue, especially in relation to the metabolism of bacteria. Certain theories may be advanced, however. Liver tissue, owing to its high glycogen content, furnishes an easily available food for the bacteria. Since B. diphtheriae does not form acid from glycogen no effect on the reaction of the medium results, even though it is rapidly attacked. Again, the tissue may absorb some of the oxygen in solution in the broth, thereby causing the diphtheria organisms to grow in thick pellicle formation on the surface in order to obtain an adequate supply of oxygen. The fact that only fresh tissue is of value indicates that this theory may be at least a partial explanation of the favorable results, for tissue which has stood for twenty-four hours in uninoculated broth has probably reached its limit of oxygen absorption. The surface of such a medium, unprotected by pellicle formation, is still in direct contact with the oxygen of the air which the broth probably continues to absorb. Finally, liver tissue may supply in extra quantities substances similar to those found by Spronk[8] in yeast cells, which he claimed to be essential to toxin production. Since glandular tissue is particularly rich in vitamins, the presence of these substances in liver tissue may have an important effect on toxin production. Perhaps no one of these theories completely explains the favorable action of liver tissue, but rather a combination of them. The presence of both glycogen and accessory substances in the tissue seems to us, however, to account for the rapid growth and regular toxin production in broth which has a favorable reaction. In a study of the hemotoxin production of streptococci we have found that there is in fresh tissue some substance of great delicacy essential for the elaboration of hemotoxin. In many respects there appears to be a similarity between the substances from which diphtheria toxin and the hemotoxin of streptococci are derived. The presence of glycogen and accessory substances in tissue seems to us, however, to be presumptive evidence that they play an important part in the rapid and regular toxin production.

No toxin has been produced by this method on a commercial scale owing to a lack of proper facilities. It is therefore impossible to state whether or not the trouble and expense of adding liver tissue to broth would be warranted under commercial conditions when large amounts of toxin must be produced. These observations, however, may be of value in suggesting new lines of attack on the question of the constitution of diphtheria toxin.

CONCLUSIONS

Diphtheria toxin can be produced regularly in broth to which pieces of sterile guinea-pig liver have been added. The medium must be inoculated immediately after the addition of the tissue.

Broth prepared with certain American peptones gives satisfactory results when enriched with liver tissue.

The most favorable initial reaction of the broth ranges from plus 0.3% to neutral to phenolphthalein.

The broth at the time of testing should have a reaction ranging from P_H 8.0 to P_H 8.3, although a favorable reaction is not the only essential for toxin production.

The addition of liver tissue reduces the necessary period of incubation.

Strains of B. diphtheriae other than Park-Williams No. 8 have produced a toxin of high potency by this method.